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Human Immunodeficiency Virus Type 1 Gag p24 Alters the Composition of Immunoproteasomes and Affects Antigen Presentation▽†

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Proteasomes are the major source of proteases responsible for the generation of peptides bound to major histocompatibility complex class I molecules. Antigens, adjuvants, and cytokines can modulate the composition and enzymatic activity of proteasomes and thus alter the epitopes generated. In the present study, we examined the effect of human immunodeficiency virus type 1 (HIV-1) p24 on proteasomes from a dendritic cell line (JAWS II), from a macrophage cell line (C2.3), and from murine primary bone marrow-derived macrophages and dendritic cells. HIV-1 p24 downregulated PA28β and the β2i subunit of the immunoproteasome complex in JAWS II cells but did not decrease the immunoproteasome subunits in macrophages, whereas in primary dendritic cells, PA28α, β2i, and β5i were downregulated. Exposure of JAWS II cells and primary dendritic cells to HIV-1 p24 for 90 min significantly decreased the presentation of ovalbumin to a SIINFEKL-specific $CD8^+$ T-cell hybridoma. The decrease in antigen presentation and the downmodulation of the immunoproteasome subunits in JAWS II cells and primary dendritic cells could be overcome by pretreating the cells with gamma interferon for 6 h or by exposing the cells to HIV-1 p24 encapsulated in liposomes containing lipid A. These results suggest that early antigen processing kinetics could influence the immunogenicity of $CD8^+$ T-cell epitopes generated.

Detection and elimination of cells infected with intracellular pathogens such as viruses or parasites are controlled by cytotoxic T lymphocytes (CTLs) that specifically recognize antigenic peptides (approximately 8 to 10 amino acids) bound to major histocompatibility complex (MHC) class I molecules on the surface of the cell (23, 43, 68, 71). It is the interaction of the T-cell receptor and the peptide-bound MHC class I molecule that determines recognition of self or foreign peptide (17, 27). Virtually all of the peptides bound to MHC class I molecules are derived from processing of endogenous proteins by the proteasome complex (22, 51). Peptides ranging between 2 and 25 amino acids in length are generated by the proteasome cleavage step (24, 52, 56, 66). These peptides are further trimmed and transported across the membrane of the endoplasmic reticulum and the *cis*-Golgi apparatus in an ATP-dependent manner by specific transporters associated with antigen processing (4, 40).

A key component in MHC class I processing is the 26S multicatalytic proteasome complex, which is thought to be responsible for generating 95% of the peptides (32). The proteasome complex is barrel shaped and consists of a catalytic 20S core and two 19S regulatory complexes that bind and unfold ubiquinated proteins (31). The 20S core consists of four stacked rings, with each ring composed of seven subunits. The two outer rings consist of seven different but homologous α subunits (α1 to α7), which bind to the regulatory complexes of the core. The two inner rings consist of seven different β subunits (β1 to β7) (25). The active centers of the constitutive proteasomes (c20S; constitutively expressed in all cells) are comprised of β1, β2, and β5 subunits (34). Immunoproteasomes are synthesized upon exposure of cells to gamma interferon (IFN-γ). Immunosubunits β1i, β2i, and β5i are incorporated into nascent proteasomes, thereby replacing their constitutive homologue β1, β2, and β5 subunits (2, 39, 55). These changes result in the formation of immunoproteasomes (i20S), which exhibit modified hydrolytic activity (1). Altered peptide fragments are thus generated (61, 64), leading to diverse epitopes being presented by the MHC class I molecule (6, 7, 41, 63). In addition, IFN-γ also induces the PA28αβ complex, which binds to the top of the 20S proteasome complex and enhances the rate of proteolytic degradation, thus creating an altered array of epitopes (38, 58, 65).

Pathogens, throughout their evolution, have devised numerous ways to evade the host's immune system in an attempt to render them invisible. The MHC class I antigen-processing pathway allows CTLs to react quickly and efficiently to clear viral infections. Since most of the CTL epitopes are derived from proteasome-mediated degradation of the antigen, it is natural for the MHC class I processing machinery to be the target of pathogens for immune evasion (35). Since adjuvants can alter the composition of the proteasomes by causing the induction of immunoproteasomes and enhancing the expression of the PA28αβ complex, it is possible that adjuvants can prevent pathogens from interfering with the MHC class I processing pathway (36, 37, 42).

Liposomal lipid A is a very potent adjuvant and induces robust humoral and cellular responses to a number of antigens, including Ebola virus, human immunodeficiency virus (HIV),

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and malaria antigens (47–50). Recently, we showed that the adjuvant lipid A when incorporated into liposomes activated macrophages, phenotypically matured dendritic cells, and caused the upregulation of immunoproteasome subunits β2i, β5i, and PA28β (59).

Human macrophages and dendritic cells are readily infected with HIV. In fact, dendritic cells are one of the first cell types to encounter HIV (60, 70). Dendritic cells can reside in mucosal membranes and act as a "Trojan horse" to help in the spread of the virus to other areas of the body. It has been reported that exposed/infected dendritic cells lead to the infection of the $CD4^+$ T-cell population in the lymphoid tissues in vivo (11, 45, 60), with the $CD4^+$ T cells becoming the main source of HIV infection and replication after the initial encounter with the dendritic cells. Since HIV has the potential to infect various cells, it can differentially alter the antigen-processing machinery of the cell and the epitopes that are generated, thus modulating the CD8 T-cell response.

Gavioli et al. (21) demonstrated that HIV type 1 (HIV-1) Tat protein modulated the proteasome composition and activity by upregulating β2i and β5i and downregulating β1i, resulting in the generation and presentation of subdominant CTL epitopes of heterologous antigens. HIV-1 Tat, Rev, and Nef are early proteins in the viral replication cycle, whereas HIV-1 Gag, Pol, and Env are late structural proteins. Gag contains four proteins, i.e., matrix protein (p17), capsid protein (p24), nucleocapsid protein (p7), and p6 protein. HIV-1 Gag is produced in vast quantities during an infection and is capable of forming virus-like particles in the absence of other viral components (14, 19, 20, 69). Gag p24, the major capsid protein of the HIV-1 virion, has been used in clinical trials as one of the components of the HIV-1 vaccine because of the high degree of sequence conservation between different isolates. It is also well known that HIV infection causes CTL escape mutants of p24, which results in a loss of binding of CTL epitopes either to MHC class I molecules or to the T-cell receptor on $CD8^+$ T cells. Mutations in CTL epitopes and escape from CTL recognition are not unique to HIV and have also been reported for other viruses (8, 26). However, the effect of impaired antigen processing and presentation of HIV-1 antigens on the generation of CTL escape mutants has not been fully elucidated.

In this study, we examined the effect of HIV-1 p24 on proteasomes and on the presentation of a heterologous antigen, ovalbumin (OVA), using both cell lines and primary antigen-presenting cells. The results indicate that exposure of the JAWS II dendritic cell line and primary dendritic cells to HIV-1 p24 reduced the ability of the cells to present OVA to a SIINFEKL-specific $CD8^+$ T-cell hybridoma. HIV-1 p24 also downregulated the PA28β and to a lesser extent the β2i subunit of the immunoproteasome complex in JAWS II cells. In primary dendritic cells, PA28α, β2i, and β5i were downregulated. The decrease in antigen presentation and the downmodulation of the immunoproteasome subunits in JAWS II cells and in primary dendritic cells could be overcome by pretreatment with IFN-γ or by encapsulating p24 in liposomes containing lipid A [L(p24 + LA)] in a time-dependent manner. However, HIV-1 p24 did not decrease the proteasome composition or the antigen presentation ability of C2.3 macrophages.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice (6 to 8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, MA) and used for the preparation of bone marrow-derived macrophages and dendritic cells. This study was conducted in compliance with the animal welfare act and adhered to the principles in the guide for the care and the use of laboratory animals. The investigators used facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The Walter Reed Army Institute of Research Animal Care and Use Committee approved all animal experiments.

Generation of primary bone marrow-derived dendritic cells and macrophages. Macrophages and dendritic cells were cultured from the marrows of C57BL/6 mice as previously described (46, 49). Dendritic cells were cultured in vitro with murine recombinant granulocyte-macrophage colony stimulating factor (0.1 mg/ml) (BioSource, Invitrogen Corporation) and interleukin-4 (IL-4) (0.04 mg/ml) (BioSource). Cells were plated at a concentration of 1×10^6 cells/well in 24-well plates (Costar; Sigma-Aldrich Corporation, St. Louis, MO) and used on day 8.

Cell lines and reagents. C57BL/6-derived JAWS II dendritic cells were obtained and grown as prescribed by the American Type Culture Collection. The C2.3 hybridoma cell line derived from C57BL/6 bone marrow-derived macrophages was kindly provided by K. L. Rock, Harvard School of Medicine, Boston, MA (33). The B3Z hybridoma cell line, specific for SIINFEKL, was kindly provided by N. Shastri, University of California, Berkeley, CA (28). Bovine serum albumin (BSA), OVA, hen egg lysozyme (HEL), and iodoacetamide were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Cycloheximide (CHX), anti-mouse β2i, and anti-mouse PA28β were purchased from Biomol (Plymouth Meeting, PA). Anti-mouse α6, β1i, β5i, PA28α, and β-actin were purchased from Abcam (Cambridge, MA). Baculovirus-expressed p24 HIV-1_{111B} was purchased from Immunodiagnostics, Inc. (Woburn, MA). *Escherichia coli*-expressed p24 and *E. coli*-expressed Soc (small outer capsid protein of bacteriophage T4) were obtained from Venigalla Rao, The Catholic University of America, Washington, DC (54). The endotoxin level of both *E. coli*-expressed and baculovirus-expressed p24 (5 μg) was ≤ 0.25 EU/ml (50 pg/ml), as measured in a *Limulus* amebocyte lysate assay. Yeast and CHO cell-expressed p24 were purchased from Vybion, Ithaca, NY, and Advanced BioSciences Laboratories, Inc., Kensington, MD, respectively. Baculovirus-expressed p24 was used in all the experiments unless stated otherwise. HIV-1 p24 was centrifuged at 14,000 rpm to remove any aggregates. ZOOM strip pH 3 to 10, protein solublizer 1, and 4 to 20% gradient gels were purchased from Invitrogen (Carlsbad, CA).

Immunofluorescence. JAWS II dendritic cells and C2.3 macrophages (1×10^6) were incubated with medium alone, *E. coli*-expressed p24 or baculovirus-expressed p24 (5 μg) in a total volume of 0.2 ml for 60 min at 37°C. Cells were washed, centrifuged onto coverslips, and then fixed and permeabilized with cold methanol for 5 min at −20°C. The nonspecific sites were blocked with 4% goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature, followed by incubation at room temperature for 1.5 h with 5 μl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-p24 (clone KC57-FITC; Beckman Coulter, Fullerton, CA) in a total volume of 1 ml of PBS. After the cells were washed three times with PBS, the coverslips containing the cells were mounted on slides with Vectashield mounting medium and sealed with nail polish. Cells were examined with a Bio-Rad Radiance 2100 confocal microscope using a 60× oil immersion objective. Data were collected using LaserSharp software. In addition, JAWS II dendritic cells were incubated in the presence and absence of 200 μg of HEL or 10 μg of *E. coli*-expressed Soc and treated as described above to visualize internalization of the antigen. Murine polyclonal anti-HEL antibodies or rabbit polyclonal anti-Soc antibodies generated in our laboratory were used as the primary antibody (1 h) and visualized with FITC-conjugated goat anti-mouse immunoglobulin G (1 h) (Thermo Scientific, Rockford, IL) or FITC-conjugated goat anti-rabbit immunoglobulin G (1 h) (Abcam, Cambridge, MA). Cells were examined with an Olympus BH2 fluorescence microscope using a 40× objective (see Fig. S1A and S1B in the supplemental material).

SDS-PAGE and Western blotting. JAWS II and C2.3 cells (1×10^6 cells) were incubated with 25 μg/ml of p24 for various time periods. Cells were then lysed in buffer (20 mM Tris [pH 7.2], 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, and 1% Triton X-100), and the samples were run on a 4 to 20% gradient gel (Invitrogen). The proteins were then electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked (PBS, 5% fat free milk powder, 0.05% Tween 20) for 1 h before an overnight incubation with a 1:5,000 dilution of polyclonal mouse anti-p24 antibody (54) in PBS containing 0.05% Tween 20. The blots were washed and incubated for 1 h with a 1:10,000 dilution of goat anti-mouse–alkaline phosphatase-labeled antibody (Promega, Madison, WI) in PBS containing 0.05% Tween 20. The blots were washed and

developed using nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate substrate (Bio-Rad). The dried blots were scanned using UMAX VistaScan software.

Flow cytometry. Flow cytometry was performed as previously described (59). Briefly, cells were collected from 6- or 24-well plates, washed in sterile ice cold PBS, and resuspended (1×10^6 cells) in flow buffer (PBS containing 1% BSA). Cells were preincubated with anti-mouse CD32/16 antibody (Fc block, 1 μg/1×10^6 cells) for 10 min at 4°C before surface staining. Cells were labeled at 4°C for 20 min with the following anti-mouse phycoerythrin-conjugated antibodies: H-2K^b, CD40, CD80, CD86, and the appropriate isotype controls (all purchased from BD Biosciences). Cells were washed twice in ice-cold flow buffer and fixed in 2% formaldehyde in PBS, and 25,000 gated events were collected for analysis on a FACScan (BD Biosciences) using Cell Quest (BD Biosciences) and Flow Jo (TreeStar, Ashland, OR). The results are expressed as histograms.

Intracellular staining for HIV-1 p24. JAWS II and C2.3 cells (1×10^6) were incubated with soluble *E. coli*-expressed p24 (25 μg/ml), or in separate experiments, JAWS II cells were incubated with *E. coli*-expressed p24 encapsulated in L(LA) for 30 min at 37°C in a CO_2 incubator. Cells were then washed, fixed, and permeabilized using the Caltag kit. Cells were stained with anti-p24–FITC monoclonal antibody clone KC57-FITC (Beckman Coulter). Cells were washed and resuspended in 1% paraformaldehyde, and 25,000 gated events were collected on a FACScan for analysis. The experiment was repeated four times with JAWS II cells and twice with C2.3 cells with duplicate samples in each case. The results are expressed as histograms.

Liposome preparation. Multilamellar liposomes were prepared as previously described (59, 67). The lyophilized liposomes were reconstituted with either PBS (pH 7.4) [L(LA)] or 1 mg/ml of p24 [L(p24 + LA)] in sterile saline for 48 h to yield a 100 mM phospholipid multilamellar liposome suspension. Liposomes were washed twice in sterile saline and reconstituted in 1 ml of sterile saline. The amount of antigen encapsulated in liposomes was determined by the modified Lowry procedure (3, 67).

Antigen presentation assays. JAWS II dendritic cells, C2.3 macrophages, and bone marrow-derived primary cells were diluted to 2×10^6 cells per ml in serum-free medium and cultured as such or in the presence of 5 μg of HIV-1 Gag p24, 5 μg of Soc, or 200 μg of HEL in a total volume of 0.2 ml for 90 min at 4°C. The antigen-pulsed cells were then washed and incubated with either 1 mg/ml of OVA or 0.1 mg/ml of SIINFEKL peptide for 90 min. Cells incubated with OVA without prior exposure to p24 served as the positive control. Alternatively, JAWS II and C2.3 cells were preincubated with CHX at a final concentration of 44.5 μM for 15 min before pulsing with the antigen. All cells were washed three times in serum-free medium and plated at 5×10^4 cells per well in a 96-well plate. The cells were then cocultured for 12 h, 18 h, and 24 h with 5×10^4 B3Z cells/well (a murine CD8$^+$ T-cell hybridoma specific for SIINFEKL). At the end of the coculture, supernatants were collected and stored at −80°C until assayed for the presence of IL-2. The amount of IL-2 in the supernatant was measured in triplicate using an IL-2 optEIA ELISA kit (BD PharMingen, San Jose, CA) according to the manufacturer's instructions. In separate experiments, cells were pretreated for 3 h or 6 h with either IFN-γ (200 units), L(LA), or L(p24 + LA) (7.6 μg p24), followed by washing and incubation for 90 min with soluble p24 (25 μg/ml). Cells were then incubated with OVA (1 mg/ml) for 90 min, washed, and then cocultured for 12 h, 18 h, and 24 h with B3Z cells as described above.

Two-dimensional (2-D) IEF. Isoelectric focusing (IEF)/SDS-PAGE was preformed as previously described (59). JAWS II, C2.3, and primary cells were incubated with 25 μg/ml of p24 (see Fig. 5 and 6 and see Fig. S3 in the supplemental material) or 25 μg/ml of Soc (JAWS II cells only; see Fig. S2B in the supplemental material) for 12 h, 18 h, or 24 h at 37°C. In separate experiments (see Fig. 9), JAWS II and C2.3 cells were preincubated for 6 h at 37°C with either medium alone, 25 μl of L(p24 + LA) (7.6 μg p24), or 200 units IFN-γ (BioSource/Invitrogen, CA) before treatment with soluble p24 (25 μg/ml) for 18 h at 37°C. Cells were washed in PBS and then lysed using protein solublizer 1. The protein concentration of each of the cell lysates was calculated by determining the absorbance at 280 nm in a spectrophotometer, using BSA as the standard. Equal amounts of proteins (10 mg/ml) were added to the ZOOM strip pH 3 to 10, and the strips were rehydrated overnight before IEF. The protein-focused strips were reduced and alkylated using iodioacetamide and then subjected to 4 to 20% gradient SDS-PAGE.

Western blotting. Proteins from the 2-D gels were electroblotted onto a polyvinylidene difluoride membrane and then blocked (PBS, 5% fat free milk powder, 0.05% Tween 20) for 1 h before overnight incubation with a mixture of anti-mouse α6, β1i, β2i, β5i, PA28α, PA28β, and β-actin in PBS containing 0.05% Tween 20. The blots were processed as described previously (59). The dried blots were scanned using UMAX VistaScan software. The scanned images of 2-D Western blots were analyzed using ImageJ software (NIH, Bethesda, MD) to determine the density of the spots. The density of each spot is expressed as arbitrary units. For each culture condition, the α6 subunit of the proteasome in the respective blot was used as the control reference spot and the β1i, β2i, β5i, PA28α, and PA28β were normalized to the corresponding α6 subunits by dividing the density of each of the specific subunits by the density of the corresponding α6 subunit (37). The inducible proteasome subunits from JAWS II, C2.3, and primary cells exposed to HIV-1 Gag p24, IFN-γ, IFN-γ plus p24, and L(p24 + LA) are graphically represented as normalized arbitrary units.

RESULTS

Internalization of HIV-1 p24 in JAWS II and C2.3 cells. To ensure that both JAWS II dendritic cells and C2.3 macrophages internalized p24, confocal microscopy and Western blot analysis of the cells were performed using *E. coli*- and baculovirus-expressed p24. HIV-1 p24 from two different expression systems was used to demonstrate that similar results were obtained irrespective of the expression system. p24 was internalized in both cell types (Fig. 1A-c, B-c, C-c, and D-c) as visualized by FITC-labeled mouse anti-p24 antibody. Cells incubated with medium and stained with FITC-labeled mouse anti-p24 antibody are shown in Fig. 1A-a, B-a, C-a, and D-a). The corresponding bright-field images are shown in Fig. 1A-b, A-d, B-b, B-d, C-b, C-d, D-b, and D-d). In Fig. 1A, the reason that not all cells appear to be staining for p24 is because the cells were observed under a confocal microscope and were sectioned at a thickness of 1 μm and only one of the sections is shown. JAWS II cells are nonadherent and are not uniform in size. Therefore, not all of the cells are in the same focal plane of the thin section to demonstrate the internalized p24.

As early as 15 min postincubation, p24 was detected in the lysates of JAWS II and C2.3 cells as determined by SDS-PAGE followed by Western blotting (Fig. 1E). HIV-1 p24 was detected up to 6 h in JAWS II cells, whereas in C2.3 macrophages, p24 was rapidly degraded and could be visualized only up to 3 h. An equal amount of p24 was internalized in both JAWS II and C2.3 cells as determined by intracellular p24 staining (Fig. 1F). The 30-min time point was chosen since macrophages rapidly degrade internalized p24. In each case, two peaks were observed. The average percentage ± standard deviation (SD) of p24-positive cells in each of the two peaks is depicted within the histogram. Both JAWS II cells and C2.3 macrophages had 87 to 88% of intracellular p24-positive cells in peak 1 and 12 to 13% in peak 2. The mean fluorescence intensity for peak 2 was 36.72 ± 5.70 for JAWS II cells and 39.30 ± 8.07 for C2.3 macrophages. Therefore, there were no significant differences in the percentage of p24-positive cells and in the uptake of p24 between the two cells types.

HIV-1 p24 does not alter cell surface markers. To determine whether p24 would alter costimulatory molecules, JAWS II and C2.3 cells were incubated overnight with p24, washed, stained, and examined by flow cytometry. As shown in Fig. 2, p24 did not alter, CD40, CD80, CD86, or MHC class I molecules on either JAWS II or C2.3 cells compared to cells treated with medium alone. The shaded histogram represents the isotype control.

HIV-1 p24 inhibits antigen presentation of OVA in JAWS II dendritic cells but not in C2.3 macrophages. Having determined that JAWS II and C2.3 cells readily internalize p24 and that p24 does not alter costimulatory markers, we then examined whether p24 would influence the antigen presentation

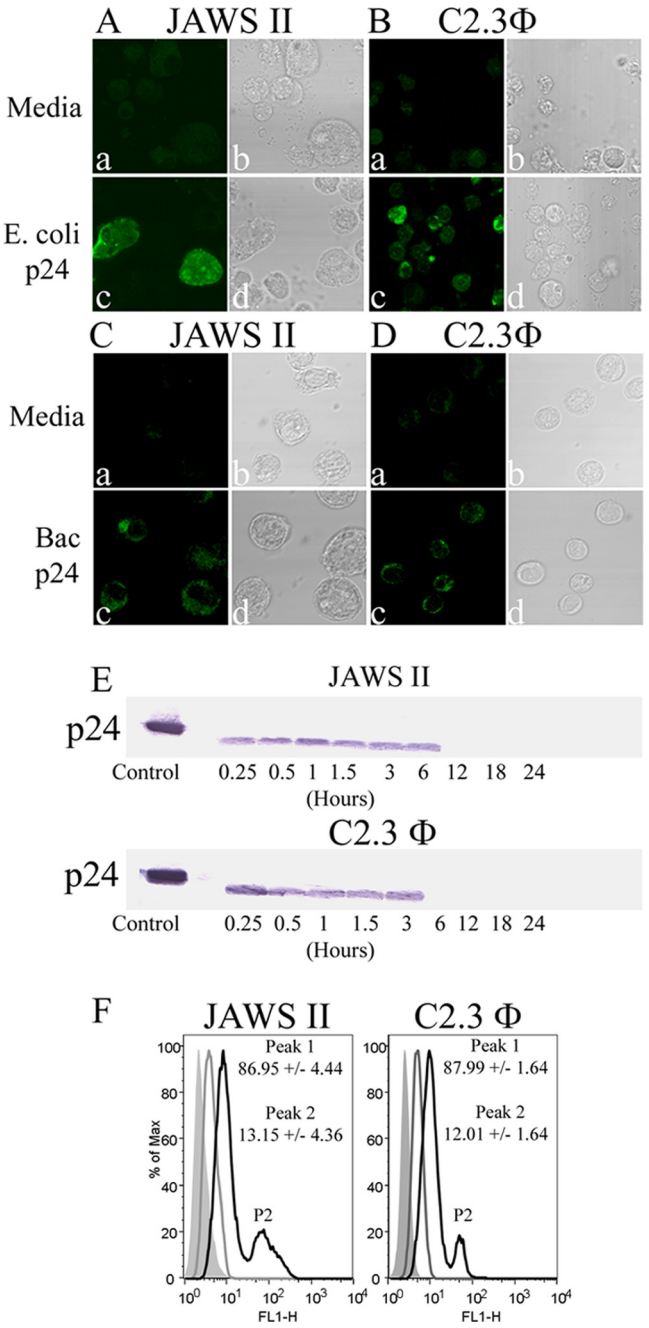

FIG. 1. Internalization of HIV-1 p24 by JAWS II cells and C2.3 macrophages. JAWS II dendritic cells (A and C) and murine C2.3 macrophages (B and D) were incubated with medium (a and b) or with 5 μg of either *E. coli*-expressed p24 (A-c and A-d) or baculovirus-expressed p24 (C-c and C-d) in a total volume of 0.2 ml for 60 min at 37°C. Cells were then washed, centrifuged onto coverslips, fixed, permeabilized, and treated as described in Materials and Methods. Cells were examined with a Bio-Rad Radiance 2100 confocal microscope using a 60× oil immersion objective. Data were collected using LaserSharp software. JAWS II and C2.3 cells incubated with medium alone showed no to very faint background staining (A-a, B-a, C-a, and D-a, respectively). JAWS II and C2.3 cells incubated in the presence of p24 showed very bright staining for p24 (A-c, B-c, C-c, and D-c, respectively), indicating that p24 was internalized in both cell types. The corresponding bright-field images are shown in panels A-b, B-b, C-b, D-b, A-d, B-d, C-d, and D-d, respectively. Western blot analysis of internalized baculovirus-expressed p24 is shown in panel E. HIV-1 p24 was incubated with either JAWS II cells (top blot) or C2.3 macrophages (bottom blot) for various time periods. Cell lysates were analyzed by Western blotting for the presence of internalized p24 by probing with murine polyclonal p24 antiserum. JAWS II and C2.3 cells internalized similar amounts of p24 at 30 min as measured by flow cytometry (F). The shaded area in the histograms represents unstained cells. The gray and black lines represent cells cultured in medium alone or in medium containing p24, respectively. The average percentage of intracellular positive p24 cells in peaks 1 and 2 ± SD are shown in each of the histograms. The data represent averages from four experiments and two experiments done in duplicate with JAWS II cells and C2.3 macrophages, respectively. The mean fluorescence intensities of peak 2 were 36.72 ± 5.70 and 39.30 ± 8.07 for JAWS II cells and C2.3 macrophages, respectively. There were no significant differences in the percentage of p24-positive cells and in the uptake of p24 in the two cell types.

capacity of these cells. The experimental setup is shown in Fig. 3A. JAWS II and C2.3 cells were pretreated with or without CHX and then pulsed with either medium, p24, or HEL for 90 min, followed by incubation with OVA or SIINFEKL peptide for an additional 90 min. The ability of JAWS II and C2.3 cells to present OVA was analyzed by coculturing the cells with B3Z cells and measuring the amount of IL-2 produced in the culture supernatants. As shown in Fig. 3B, there was a significant decrease in the ability of JAWS II cells to present OVA to B3Z cells at 12 h (39% decrease, $P < 0.0004$) and at 18 h (44% decrease, $P < 0.0001$) postculture with B3Z cells, with the antigen presentation capability returning to control levels at 24 h. Incubation of JAWS II cells with 1 mg/ml HEL (40-fold excess compared to p24) in the absence of p24, followed by OVA, did not result in any reduction in the ability of JAWS II cells to present OVA. The existence of MHC class I K^b restricted T-cell epitopes for both p24 and HEL has been previously reported (10, 18). Therefore, the decrease in OVA presentation in the presence of p24 was not due to competition for MHC class I molecules.

A significant decrease in the ability of JAWS II dendritic cells to present OVA was seen with *E. coli*-expressed p24 (see Fig. S2A in the supplemental material) at 12 and 18 h, as was observed with baculovirus-expressed p24 (Fig. 3B). Incubation of JAWS II cells with *E. coli*-expressed Soc did not cause any significant decrease in presentation of OVA at the time points tested (see Fig. S2A in the supplemental material). This was not due to a lack of internalization of the control proteins (see Fig. S1A and S1B in the supplemental material), thus demonstrating the specificity of p24 and eliminating the possibility of contaminants being responsible for the reduction in OVA presentation.

In order to determine if p24 was negatively influencing either the antigen processing or the presentation, cells were treated with CHX to prevent de novo protein synthesis before the addition of p24 or HEL followed by OVA. CHX should not affect the composition of the preformed proteasomes within the cells. The ability of JAWS II cells exposed to p24 or HEL to present OVA was maintained at control levels following the addition of CHX, although the presentation was lower than that by untreated cells (Fig. 3B). However, there was no difference in OVA presentation in cells incubated with p24 or HEL compared to medium alone. Therefore, the decrease in

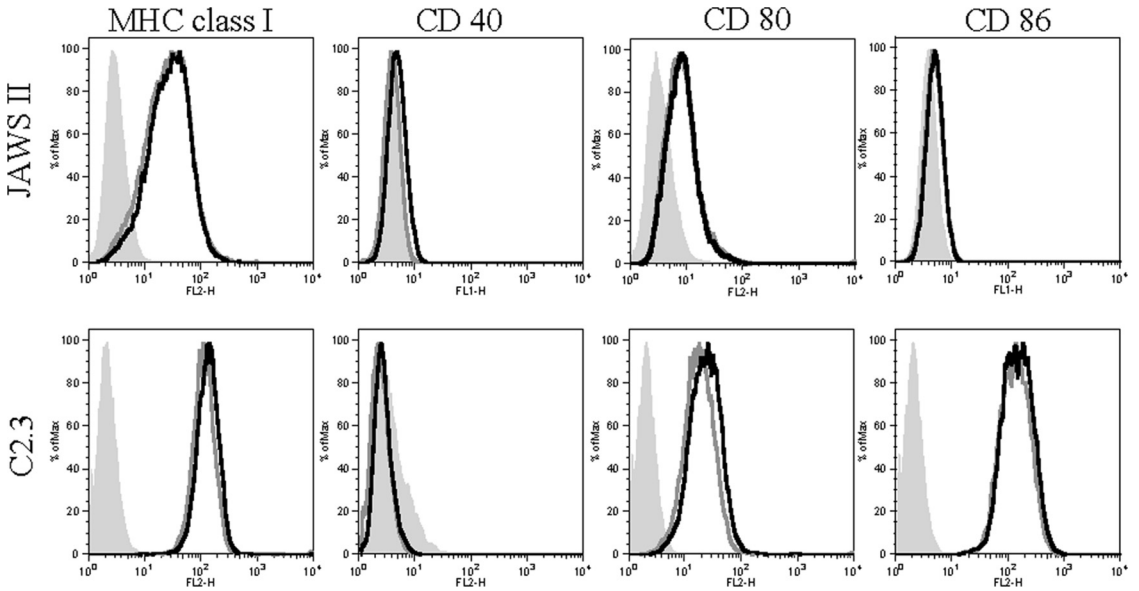

FIG. 2. Expression of cell surface molecules on JAWS II cells and C2.3 macrophages following incubation with p24. Cells were cultured in medium (gray line) or in medium containing 25 μg/ml p24 (black line) for 90 min, washed, and then incubated overnight for 18 h at 37°C. Cells were then stained for CD40, CD80, CD86, and MHC class I and analyzed by flow cytometry. The shaded area represents the isotype controls. Results from a representative of two separate experiments are shown.

OVA presentation in the presence of p24 (Fig. 3B) was due to interference in one of the components of the antigen-processing machinery. In separate experiments, JAWS II and C2.3 cells were pulsed with SIINFEKL (MHC class I epitope of OVA) and then cocultured with B3Z cells to ensure that CHX-treated cells did not affect the production of IL-2 from B3Z cells due to a carryover of CHX. There was no significant difference in the amount of IL-2 produced in both JAWS II and C2.3 cells that were either pretreated or not pretreated with CHX (Fig. 3C).

The overall antigen presentation capacity of C2.3 macrophages was higher than that observed with JAWS II dendritic cells. Antigen presentation in C2.3 macrophages incubated with p24 was not significantly altered compared to that in control cells at the 12-h and 18-h time points. However, there was a significant increase in OVA presentation compared to control cells at the 24-h time point in cells incubated with either p24 or HEL (Fig. 3B, $P < 0.00002$). As was observed in JAWS II cells, there was no alteration in OVA presentation in the presence of CHX compared to the control.

HIV-1 p24 inhibits antigen presentation of OVA in primary dendritic cells but not in primary macrophages. To rule out the possibility that the decrease in OVA presentation in the JAWS II dendritic cell line incubated with p24 was not restricted to this particular cell line, the effect of p24 on bone marrow-derived primary dendritic cells and macrophages was examined. As shown in Fig. 4, p24 decreased the ability of primary dendritic cells to present OVA at both 12 h and 18 h. These results were similar to those observed with the JAWS II cell line (Fig. 3B). In primary macrophages, p24 caused a significant enhancement of antigen presentation at all three time points tested ($P \leq 0.01$). Therefore, it appears that p24 inhibits antigen presentation in dendritic cells, whereas it causes enhancement of antigen presentation in macrophages.

HIV-1 p24 affects proteasome composition and downregulates PA28β in JAWS II cells. To examine the effect of Gag p24 on the proteasome composition of JAWS II dendritic cells, the cell lysates from unstimulated cells (medium) and cells exposed to p24 were subjected to 2-D gel electrophoresis followed by Western blotting (Fig. 5A). The dried blots were scanned using UMAX VistaScan software. The scanned images of 2-D Western blots were analyzed using ImageJ software (NIH, Bethesda, MD). A surface plot of the 2-D scanned Western blots (Fig. 5B) was used to quantify the density of each of the subunits as described in Materials and Methods. The inducible proteasome subunits from JAWS II dendritic cells exposed to medium (unstimulated) and p24 are graphically represented as normalized arbitrary units (Fig. 5C). In all cases, equivalent amounts of the protein extracts were loaded as verified by the loading control β-actin (Fig. 5D).

Analysis of JAWS II cells exposed to p24 showed a decrease in the intensity of the β2i spot at 12 h and 18 h after a 90-min exposure to p24 compared to that in unstimulated cells (Fig. 5C). A 2.2- to 3-fold decrease in the β2i subunit at 12 h and 18 h, respectively, was observed (Fig. 5C). One of the major proteasome components that appeared to be conspicuously low to absent at the 12-h and 18-h time points was PA28β (Fig. 5A to C). There was a sixfold decrease in PA28β subunit at each of these time points (Fig. 5C). The normalized arbitrary units for two separate experiments are presented in Table S1 in the supplemental material. The decrease in β2i and PA28β subunits was not restricted to baculovirus-expressed p24. Similar results were obtained with *E. coli*-, yeast-, and CHO-expressed p24 (see Fig. S2B in the supplemental material). Incubation of JAWS II cells with *E. coli*-expressed Soc for 12 h, 18 h, and 24 h did not decrease the immunoproteasome subunits (see Fig. 2C in the supplemental material), thus demonstrating the specificity of *E. coli*-expressed p24.

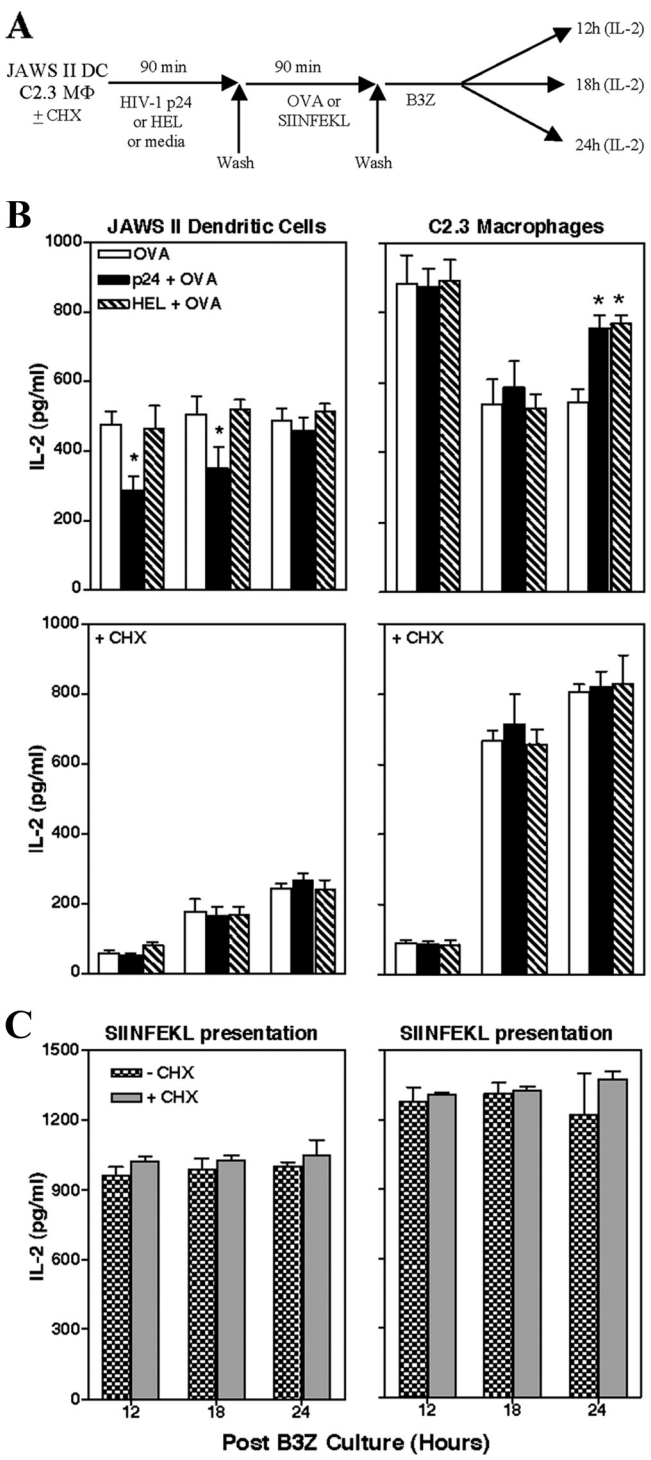

FIG. 3. Decreased ability of JAWS II cells to present OVA following exposure to HIV-1 p24. (A) Experimental setup. (B) Ability of JAWS II cells and C2.3 macrophages to present OVA as measured by IL-2 production. JAWS II cells incubated with p24 followed by OVA showed a significant decrease in IL-2 production at 12 h and 18 h compared to OVA alone (no p24). There was no decrease in IL-2 production in the presence of a nonviral antigen, HEL. In separate experiments, JAWS II cells and C2.3 macrophages were preincubated with 44.5 μM of CHX to prevent de novo protein synthesis, followed by the addition of p24 or HEL. CHX did not affect the ability of JAWS II cells to present OVA as indicated by no significant difference in IL-2 production compared to CHX-treated cells incubated with OVA alone. There was no decrease in the antigen presentation capacity of C2.3 cells in the presence of p24, HEL, or preincubation with CHX. (C) Ability of JAWS II cells and C2.3 macrophages to present OVA peptide SIINFEKL as measured by IL-2 production. CHX did not alter antigen presentation in either JAWS II cells or C2.3 macrophages compared to CHX-untreated cells. Each bar represents the mean ± SD of five replicate measurements, and results from one representative experiment out of three are shown. *, significant values compared to the control ($P < 0.0004$).

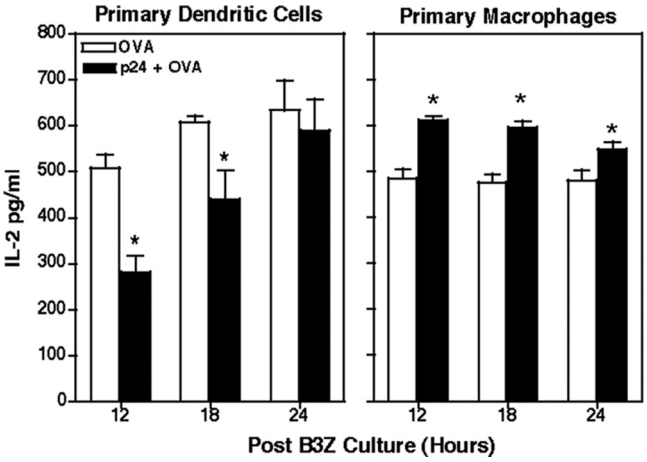

FIG. 4. Decreased ability of primary bone marrow-derived dendritic cells to present OVA following exposure to HIV-1 p24. The experimental setup is shown in Fig. 3A. The ability of primary dendritic cells and macrophages to present OVA as measured by IL-2 production is shown. Primary dendritic cells incubated with p24 followed by OVA showed a significant decrease in IL-2 production at 12 h and 18 h compared to those incubated with OVA alone (no p24) ($P < 0.0008$). There was no decrease in the antigen presentation capacity of primary macrophages in the presence of p24, but an enhancement was observed ($P \leq 0.01$). Each bar represents the mean ± SD of triplicate measurements, and results from one representative experiment out of two are shown. *, significant values compared to the control.

Unlike the JAWS II cells, which express all immunoproteasome subunits (β1i, β2i, β5i, PA28α, and PA28β) under normal conditions, primary dendritic cells express all immunoproteasome subunits at lower levels, except for PA28β, which is absent and can be induced upon stimulation with cytokines. Studies carried out with primary dendritic cells indicated that p24 affected the immunoproteasome subunits β2i as observed with the JAWS II cells and, additionally, β5i as well as the PA28α subunit (see Fig. S3 in the supplemental material).

HIV-1 Gag p24 does not downregulate β2i and PA28β in C2.3 macrophages. To examine the effect of p24 on the proteasome composition of C2.3 macrophages, the cell lysates from unstimulated cells (medium) and cells exposed to p24 were subjected to 2-D gel electrophoresis followed by Western blotting (Fig. 6A). The Western blots were analyzed as described above using ImageJ software, and the results are represented graphically as described above. In contrast to what was observed with JAWS II cells, p24 did not cause the downregulation of any of the immunoproteasome subunits in the macrophage cell line at 12 h or at 18 h (Fig. 6B). In fact, an enhancement of the PA28β subunit was observed (Fig. 6A and

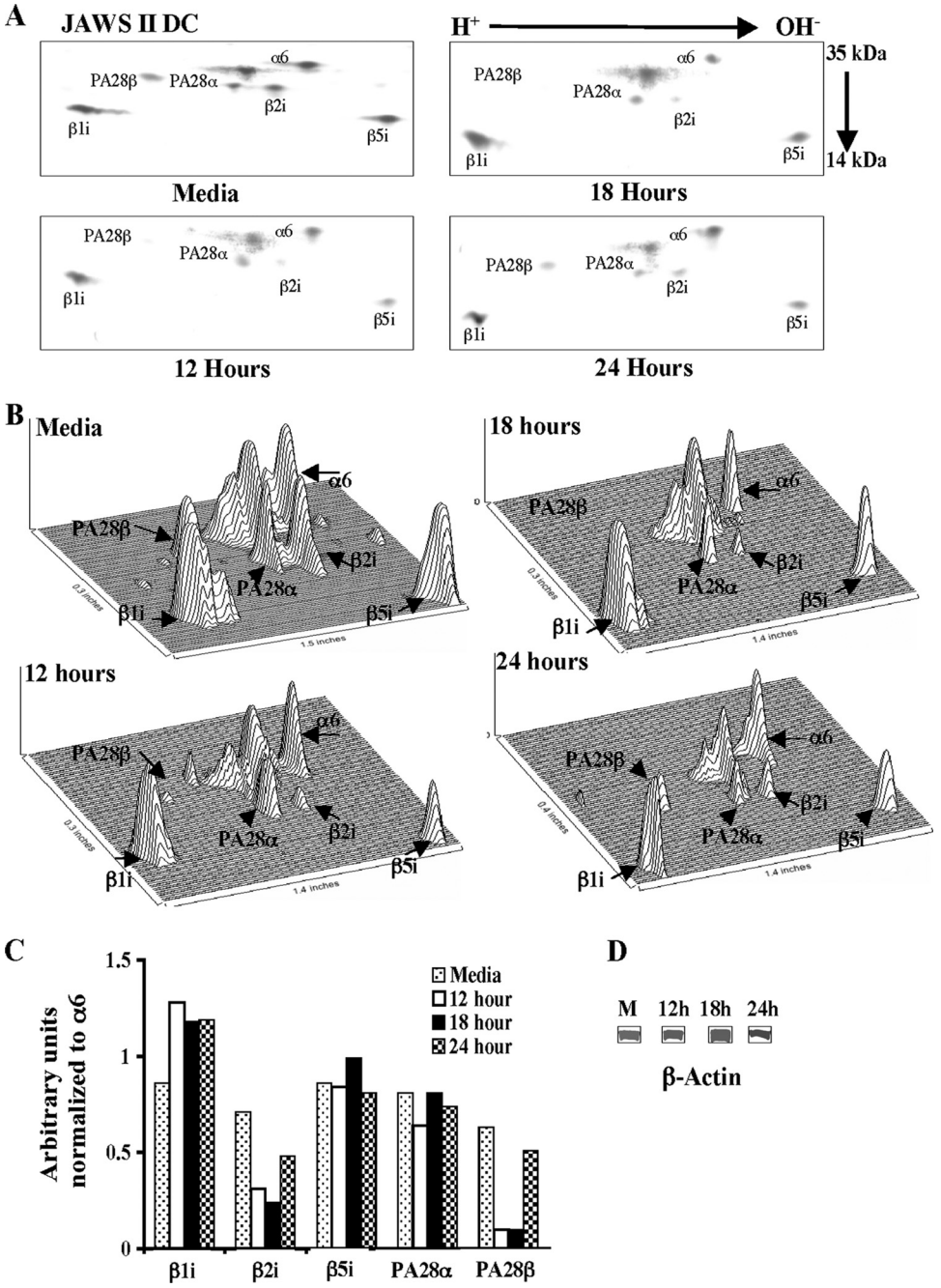

FIG. 5. Analysis of the immunoproteasome subunits from JAWS II cells following incubation with HIV-1 p24. JAWS II cells were incubated with HIV-1 p24 for 90 min in serum-free medium, washed, and then put back in culture for 12 h, 18 h, or 24 h. Cells cultured in medium without baculovirus-expressed p24 for the entire period served as the control. Cell lysates were subjected to 2-D gel electrophoresis followed by Western blotting (A). The blots were probed with a mixture of anti-mouse α6, β1i, β2i, β5i, PA28α, PA28β, and antibody to β-actin. The dried blots were then scanned and analyzed using ImageJ software (NIH, Bethesda, MD) to determine the density of the spots. The middle panels (B) represent surface plots of each of the 2-D scanned Western blots that were then used to quantify the density of each of the subunits. The density of the spots is expressed as arbitrary units (C). For each culture condition, the α6 subunit of the proteasome in the respective blot was used as the control reference spot, and the β1i, β2i, β5i, PA28α, and PA28β subunits were normalized to the corresponding α6 subunit by dividing the density of each of the specific subunits by the density of the corresponding α6 subunit. The inducible proteasome subunits from JAWS II cells exposed to medium or incubated with p24 for 12 h, 18 h, or 24 h are graphically represented as normalized arbitrary units in panel C. In all cases, equivalent amounts of the protein extracts were loaded as verified by the loading control β-actin (D). Results from one representative experiment out of two are shown.

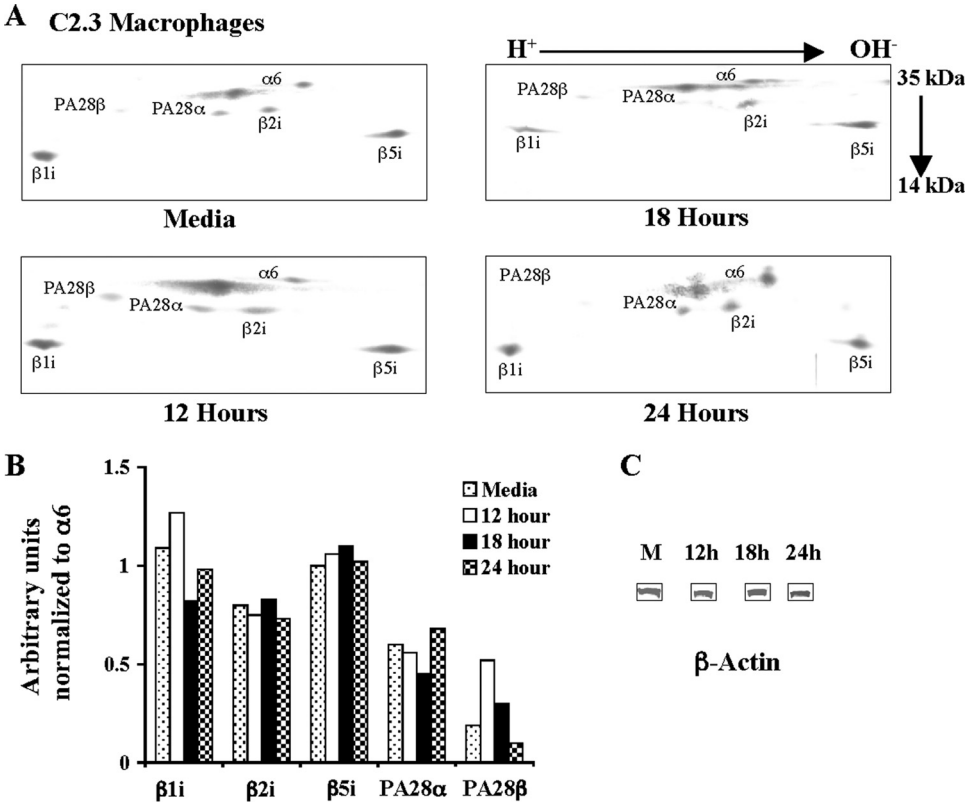

FIG. 6. Analysis of the immunoproteasome subunit from C2.3 cells following incubation with HIV-1 p24. For details, see the legend to Fig. 5. Results from one representative experiment out of two are shown.

B). In all cases, equivalent amounts of the protein extracts were loaded as verified by the loading control β-actin (Fig. 6C). The normalized arbitrary units for two separate experiments are presented in Table S1 in the supplemental material.

The antigen presentation ability of JAWS II dendritic cells exposed to HIV-1 p24 is restored in a time-dependent manner by preexposure of cells to IFN-γ or to L(p24 + LA). We have recently demonstrated that L(LA) caused the upregulation of immunoproteasome subunits β2i, β5i, and PA28β (59). Using macrophages and dendritic cells generated from IFN-γ knockout mice, we also showed that the maturation of dendritic cells, activation of macrophages, and induction of immunoproteasomes by L(LA) were through the production of the immunomodulatory cytokine IFN-γ (59). In the present study, the decrease in antigen presentation observed with JAWS II cells (Fig. 3B) and primary dendritic cells (Fig. 4) incubated with p24 could be reversed by preexposing the cells either to IFN-γ (Fig. 7A, B, and D) or to L(p24 + LA) (Fig. 7C and E). The control consisted of cells exposed to either IFN-γ and OVA or L(LA) and OVA in the absence of p24 (Fig. 7). The time for reversal of antigen presentation for JAWS II cells pretreated with IFN-γ was 6 h (Fig. 7B). However, this was not the case with primary dendritic cells (Fig. 7D). When JAWS II cells were preincubated with L(p24 + LA) (Fig. 7C) for either 3 h or 6 h followed by soluble p24, antigen presentation was again restored to normal levels, whereas primary dendritic cells required at least a 6 h of pretreatment (Fig. 7E). The decrease in antigen presentation observed with soluble p24 (Fig. 3B) and the restoration by L(p24 + LA) was not due to internalization of different amounts of soluble and liposome-encapsulated p24, since flow cytometry analysis showed equal mean fluorescence intensities in both cases (see Fig. S4 in the supplemental material).

In contrast, C2.3 macrophages did not show a decrease in the antigen presentation with p24 (Fig. 3B). Pretreatment of C2.3 macrophages with either IFN-γ (Fig. 8A) or L(p24 + LA) (Fig. 8B) did not result in a decrease in presentation of OVA to B3Z cells. In fact, a significant enhancement was seen.

IFN-γ and L(p24 + LA) restore the immunoproteasome composition in JAWS II dendritic cells. To examine if the rescue in antigen presentation was due to the restoration of the immunoproteasome subunits, 2-D gel electrophoresis followed by Western blotting was performed on the lysates of JAWS II or C2.3 cells (Fig. 9) that were either unstimulated, stimulated with IFN-γ, stimulated with IFN-γ followed by the addition of p24, or incubated with L(p24 + LA) (Fig. 9A and B). The intensity of the PA28β immunoproteasome subunit spot from cell lysates of JAWS II and C2.3 cells was not altered with the various treatments and was similar to that observed with unstimulated cells (Fig. 9A). The scanned images of 2-D Western blots were analyzed using ImageJ software as previously described for Fig. 5. No significant decrease in the immunoproteasome subunits was observed for either JAWS II or C2.3 cells incubated with L(p24 + LA) compared to cells stimulated with IFN-γ (Fig. 9B). In all cases, equivalent amounts of the protein extracts were loaded as verified by the loading control

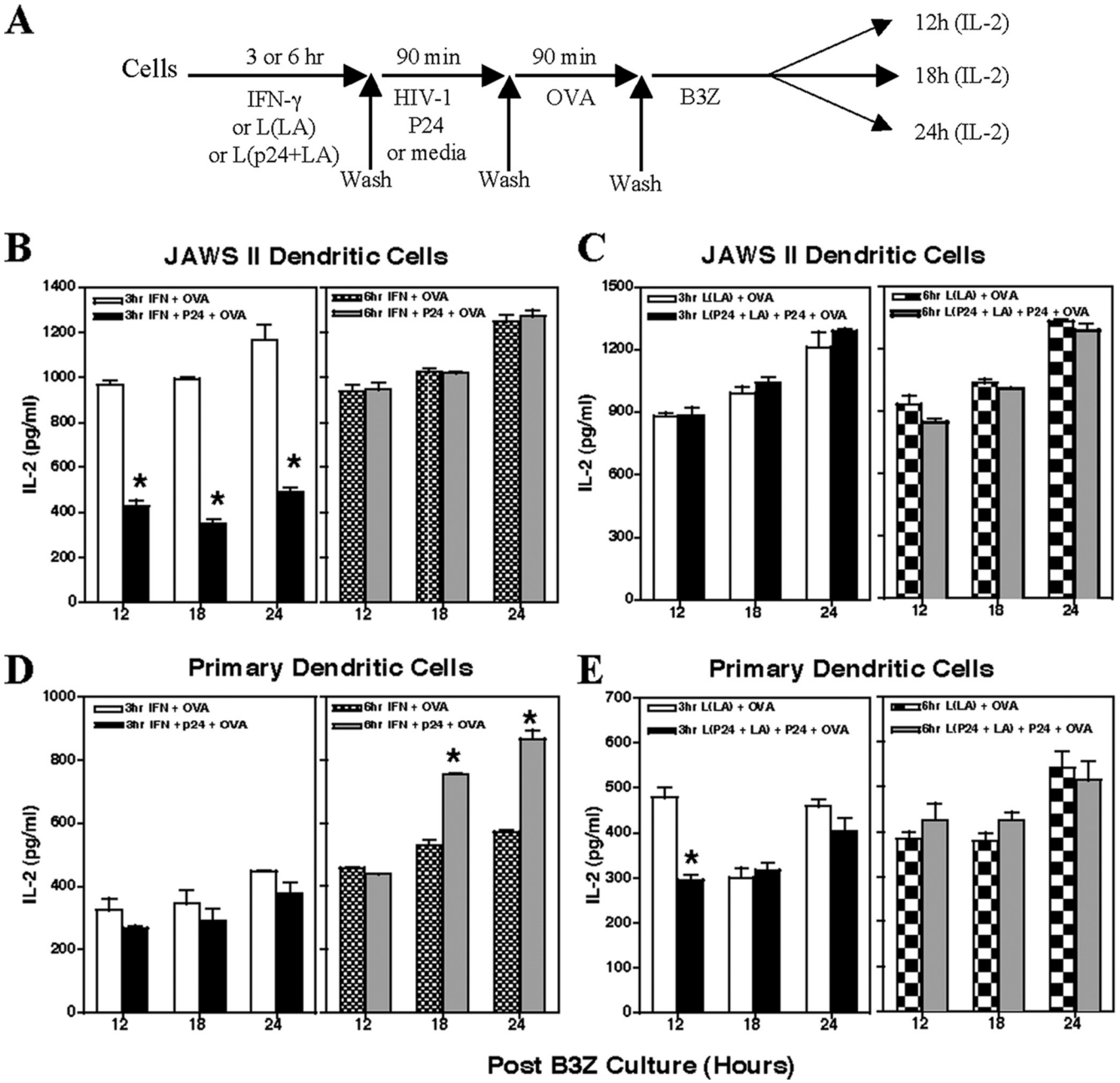

FIG. 7. Treatment with IFN-γ or L(p24 + LA) restores the antigen-presenting ability of JAWS II and primary dendritic cells. (A) Schematic diagram of treatment of antigen-presenting cells. (B to E) JAWS II cells (B and C) or primary dendritic cells (D and E) were pretreated with IFN-γ (B and D) or L(p24 + LA) (C and E) for 3 h or 6 h, followed by incubation with p24 before the addition of OVA and B3Z cells. The amount of IL-2 secreted was measured at 12 h, 18 h, and 24 h after B3Z culture. The respective controls consisted of cells treated with IFN-γ or L(LA) for 3 h or 6 h followed by incubation with OVA in the absence of p24. The data are depicted as the amount of IL-2 produced in pg/ml. Pretreatment of JAWS II and primary dendritic cells for 6 h with IFN-γ or L(p24 + LA) restored or increased the ability of cells to present OVA at control or higher levels in the presence of p24. Each bar represents the mean ± SD of triplicate measurements. Results from one representative experiment out of two are shown. *, significant values compared to the control ($P < 0.0001$).

β-actin (Fig. 9C). These results indicate that the downregulation of PA28β induced by p24 (Fig. 5A) can be reversed by the addition of IFN-γ or by encapsulating p24 in liposomes containing lipid A (Fig. 9B), thereby restoring the ability of the cells to process and present antigens at normal levels (Fig. 7B). The normalized arbitrary units for two separate experiments are presented in Table S2 in the supplemental material.

DISCUSSION

Antigen presentation is critical both for priming an immune response and for stimulating the preprimed memory cells to defend the host against the invading pathogen. Efficient processing and presentation of a wide variety of epitopes are required for optimal stimulation of the cellular immune re-

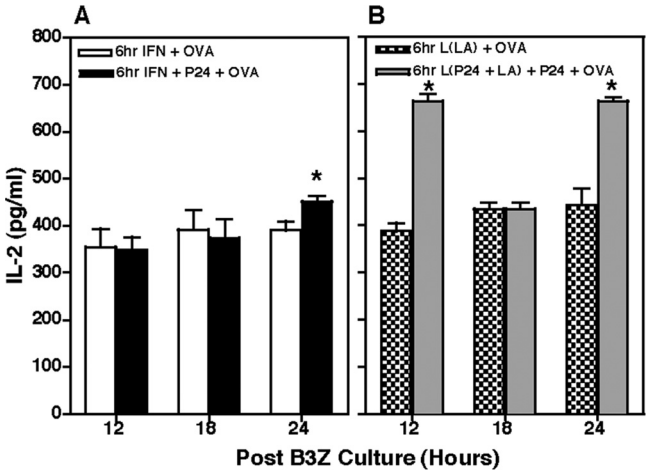

FIG. 8. Treatment with IFN-γ or L(p24 + LA) enhances the antigen-presenting ability of C2.3 macrophages. C2.3 macrophages were pretreated for 6 h with either IFN-γ (A) or L(p24 + LA) (B) as described in Fig. 7A. The amount of IL-2 secreted (pg/ml) was measured at 12 h, 18 h, and 24 h after B3Z culture. The respective controls consisted of cells treated with IFN-γ or L(LA) for 6 h followed by incubation with OVA in the absence of p24. Pretreatment of C2.3 macrophages enhanced the ability of cells to present OVA in the presence of p24. Each bar represents the mean ± SD of triplicate measurements. Results from one representative experiment out of two are shown. No decrease in IL-2 production was seen in C2.3 macrophages. *, significant values compared to the control ($P < 0.001$).

sponse and the successful clearance of the pathogen. Pathogens have devised numerous mechanisms to interfere with antigen processing and presentation. One of the organelles that is targeted by the pathogen is the proteasome complex (21, 30). Intracellular pathogens are capable of interfering with the incorporation of immunoproteasome subunits (21, 29) as a means of forcing the immune system to select specific T-cell epitopes that are less detrimental to the intracellular pathogen. This in turn could lead to the evasion of the host immune response and survival of the pathogen.

In this study, we demonstrate that in both the JAWS II dendritic cell line and the primary dendritic cells, HIV-1 Gag p24 interfered with the immunoproteasome complex by downregulating either the PA28β or the PA28α subunit and to some extent the β2i subunit, thus causing a decrease in the antigen presentation capacity of these cells. However, there was no change in the overall expression of costimulatory molecules or MHC class I molecules on the JAWS II cell surface after exposure to p24 (Fig. 2). The negative effects of p24 on the immunoproteasomes in dendritic cells were reversed by the addition of IFN-γ or L(p24 + LA) (Fig. 7 and 9). Restoration of antigen presentation was time dependent on the preexposure of cells to IFN-γ or to L(p24 + LA) (Fig. 7). Pretreatment of JAWS II cells with IFN-γ for 6 h but not for 3 h was sufficient to restore the antigen presentation capacity of the cells. This differential effect was not observed with primary dendritic cells, although antigen presentation was reduced compared to that for the control after a 3-h pretreatment. However, there was a significant enhancement of antigen presentation with 6 h of preexposure to IFN-γ. The enhancement of antigen presentation could be due to the rapid induction of immunoproteasome subunits and costimulatory molecules following pretreatment of primary dendritic cells with IFN-γ. Unlike primary dendritic cells, the JAWS II dendritic cell line has endogenous immunoproteasomes and low constitutive levels of costimulatory molecules, requiring a longer time period with IFN-γ for optimal antigen presentation. In contrast, 3 h of pretreatment of JAWS II cells with L(p24 + LA) was sufficient to restore antigen presentation. The overall results were similar in primary dendritic cells, with a 6-h pretreatment with L(p24 + LA) inducing consistent antigen presentation compared to the control cells.

We recently demonstrated that L(LA) induced the production of IFN-γ and caused a shift in the proteasome profile from constitutive to immunoproteasomes in murine bone marrow-derived macrophages and dendritic cells (59). In view of this, the restoration of the antigen presentation capacity of primary dendritic cells and JAWS II cells by L(p24 + LA) was presumably due to the production of IFN-γ (59). Interestingly, the negative effects of p24 were not seen in the C2.3 macrophage cell line or primary macrophages. Only dendritic cells can cross-present soluble antigens, whereas both macrophages and dendritic cells can cross-present particulate antigens (9, 44, 53). Also, it has been reported by Delamarre et al. (13) that the lysosomal protease content in macrophages is markedly different from that in dendritic cells, resulting in a rapid degradation of internalized antigens in macrophages, whereas the slow degradation in dendritic cells contributes to cross-presentation. Although p24 did not upregulate all the immunoproteasome subunits in C2.3 macrophages, there was a slight increase in the PA28β subunit and a significant enhancement of OVA presentation at 24 h. A significant enhancement in presentation of OVA by primary macrophages was also observed at the three time points tested (Fig. 4). The differences observed between dendritic cells and macrophages could be due to the differences in the intracellular fates of antigens in the two types of antigen-presenting cells.

Antigen presentation kinetics are crucial for the generation of an early $CD8^+$ T-cell response. An epitope that is presented early on after an infection on a professional antigen-presenting cell has a greater chance of binding naive $CD8^+$ T cells than an epitope that is presented later. Therefore, the effect of immunoproteasomes on the antigen kinetics is more important than their effect on the overall epitope quantity (15). In the present study, the antigen presentation abilities of JAWS II cells and primary dendritic cells were impaired after a 90-min incubation with p24 followed by a 90-min chase. A decrease in presentation of processed OVA as measured by IL-2 production from B3Z cells was evident at 12 h and 18 h. By 24 h the presentation ability was restored to normal levels. Consistent with the decrease in antigen presentation, there was also a decrease in the PA28β subunit and a slight decrease in the β2i subunit in the case of JAWS II cells and PA28α, β2i, and β5i in the case of primary dendritic cells. The decrease probably occurs at a preprotein level as indicated by the CHX data. The decrease in the PA28α or PA28β subunit is perhaps one potential mechanism employed by HIV to evade the host's immune response, since it has been demonstrated that generation of specific epitopes requires the presence of both the homologous α and β subunits of the PA28αβ complex (58, 62, 65).

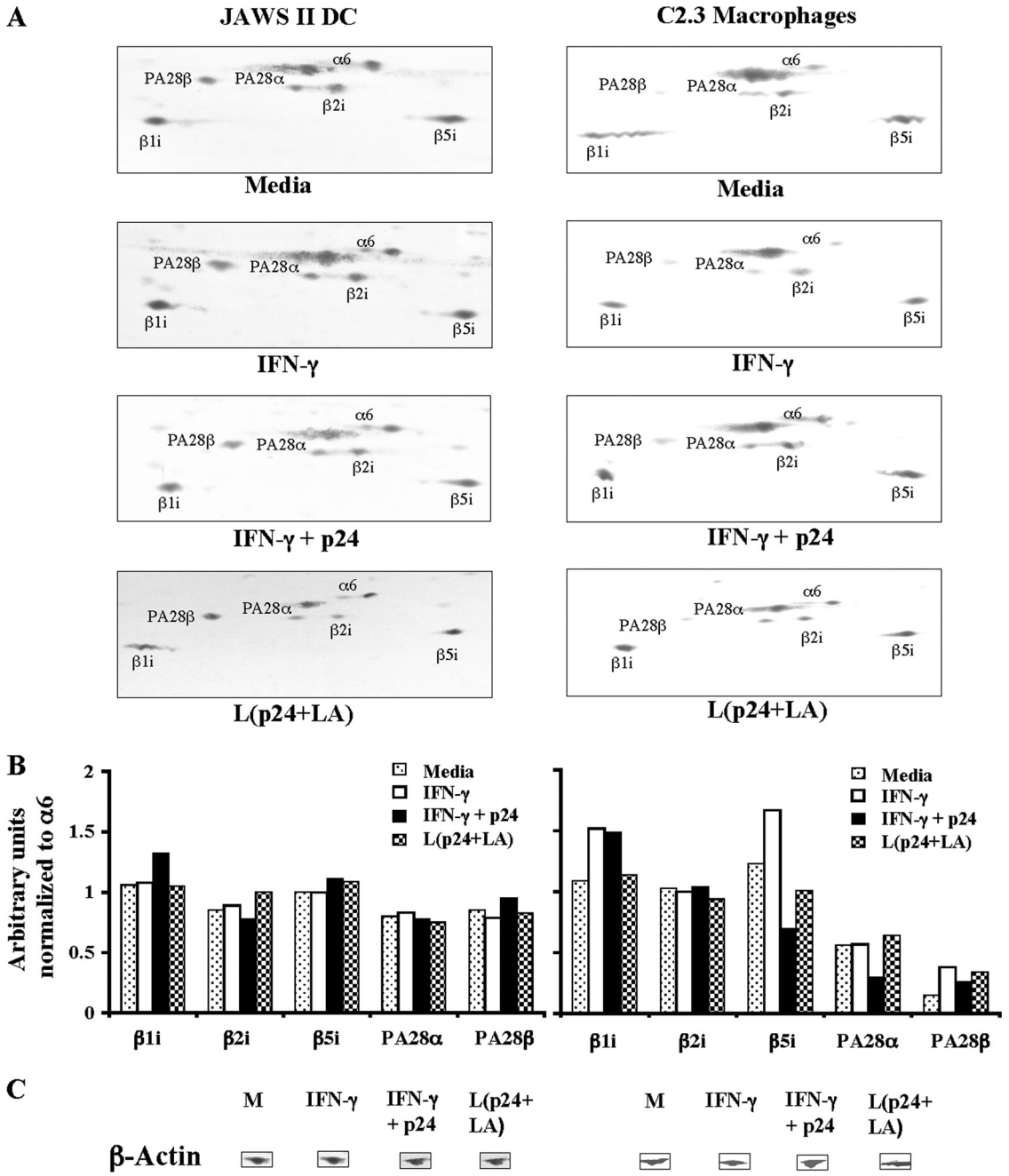

FIG. 9. Restoration of the immunoproteasome subunits in JAWS II cells following various treatments. (A) Cells were treated with medium, IFN-γ, IFN-γ followed by p24, or L(p24 + LA). Cell lysates were treated as described in Materials and Methods. (B) The densities of the proteasome subunits spots are graphically represented as normalized arbitrary units. See the legend to Fig. 5 for details. (C) In all cases, equivalent amounts of the protein extracts were loaded as verified by the loading control β-actin. Results from one representative experiment out of two are shown.

The PA28αβ complex binds to the top of the 20S proteasome complex and enhances the rate of peptide production.

Sijts et al. (57) have demonstrated that IFN-γ inducible subunits, when present at relatively low levels during the initial stages of an infection, increase the efficiency of antigenic peptide generation and thereby enhance MHC class I antigen processing in infected cells. Differential processing of antigenic peptides by proteasomes and immunoproteasomes is an import factor contributing to the immunodominance hierarchy of $CD8^+$ T-cell responses. In an lymphocytic choriomeningitis virus infection, processing of the protein by immunoproteasomes resulted in the generation of the dominant epitope as the major epitope compared to the subdominant epitope, which was poorly produced. Therefore, the peptides that are presented on the cell surface are dependent upon the type of proteasome present inside the cell, which influences the hierarchy of the CD8 T-cell responses (12, 64).

It has been previously demonstrated that the creation of

antigen-specific $CD8^+$ T-cell epitopes is dependent upon the composition and the activation of the immunoproteasomes in the cell (6). Three types of epitopes can potentially be generated: epitopes specific to constitutive proteasomes, epitopes specific to immunoproteasomes, and common epitopes created by both constitutive proteasomes and immunoproteasomes. The PA28αβ cap binds to the α subunit ring, enhancing the substrate affinity and the release of peptide products (16, 24). However, the enzymatic activity of the core complex is not altered. HIV-1 Tat protein has been reported to interact with the α4 and α7 subunits of the proteasome and to compete with the PA28αβ complex for association with the proteasome, thereby decreasing the proteasomal activity and hence the efficiency of antigen presentation (5).

Two other immunoproteasome subunits affected by Gag p24 were β2i and β5i. Immunoproteasome subunits β1i and β5i have been linked to the expansion of T-cell receptor V_β T-cells. Recent work by Bassler et al. (7) demonstrated an altered T-cell repertoire, a 20% decrease in the number of cells in the spleens of β2i knockout mice, and a reduction in the expansion of T-cell receptor $V_\beta 10^+$ T cells compared to those in wild-type mice. Furthermore, HIV infection can potentially affect various target cells in different ways. Gavioli et al. (21) demonstrated that biologically active Tat caused a reduction in the β1i subunit from T- and B-cell lines. However, our results show that Gag p24 induced this subunit in JAWS II dendritic cells. This indicates that the HIV antigens may have different effects on different cell types, leading to altered antigen processing and presentation.

In conclusion, our studies demonstrate that the composition of immunoproteasomes in the JAWS II dendritic cell line and primary dendritic cells is altered by Gag p24, with a reduction in the PA28β and β2i subunits in JAWS II cells and of PA28α (unstimulated primary dendritic cells lack the PA28β subunit), β2i, and β5i in primary dendritic cells. Concomitant with this, a decrease in heterologous antigen presentation was observed at early time points. The decrease in antigen presentation and the reduction of the immunoproteasome subunits could be restored in a time-dependent manner by pretreating the cells with IFN-γ or by encapsulating p24 in liposomes containing lipid A. These results substantiate the significance of understanding how viral antigens influence antigen processing and presentation and the importance of adjuvants. These are critical aspects to consider in the design and formulation of potential vaccine candidates.

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